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Epidemiology of Extended-Spectrum β-Lactamase-producing *Escherichia coli*

Sofia Ny

Karolinska Institutet

Stockholm 2019

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Epidemiology of Extended-Spectrum β-Lactamase-producing Escherichia coli
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To all the chickens

ABSTRACT

Extended-Spectrum β-Lactamase and plasmid mediated AmpC (ESBL/pAmpC)-producing *Escherichia coli* (*E. coli*) has during the last decades emerged worldwide and is now an increasing problem in both human and animal health. In order to slow down the spread it is important to study success factors and transmission routes so that preventive measures can be efficient.

In paper **I** we studied what sectors that contribute to human carriage and human clinical infections by investigating the molecular epidemiology of ESBL/pAmpC-producing *E. coli* in leafy greens, meats, farm animals, human community carriers and human blood stream infections. We found that different ESBL/pAmpC-genes, plasmids and *E. coli* MLST lineages dominated in isolates from humans compared to isolates in farm animals, foods and meats, but some traits overlapped. All in all, we concluded that a very small proportion of human bloodstream infections with ESBL/pAmpC-producing *E. coli* could have originated from the foods we consume.

To better understand the prevalence of ESBL/pAmpC-producing *E. coli* in the community we performed two carrier studies described in papers **II** and **III**. In paper **II** we found that 4.7% of the Swedish population carried ESBL/pAmpC-producing *E. coli* in their intestine. Risk factors associated with carriage was travel to countries in Asia and Africa and a diet that did not include pork. In paper **II** we also explored which *E. coli* populations that accumulated in clinical infections compared to carriers and found that the ESBL-gene $bla_{\text{CTX-M-15}}$ and *E. coli* ST131 and its subclone H30-Rx/C2 were overrepresented in bloodstream infections.
In paper **III** we joined forces with our neighbouring countries around the Baltic Sea, Finland, Latvia, Russia, Poland and Germany to investigate the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* in specific populations in all participating countries. We found large differences in prevalence between countries with the highest in Russia (23.4%) and the lowest in Latvia (1.6%). No carbapenemase producing isolates were identified in any of the investigated countries.

In paper **II** *E. coli* ST131 was identified as the most common ST to cause bloodstream infections in Swedish patients. This lineage is internationally wide-spread and commonly cause severe infections. In paper **IV** we explored the Swedish epidemiology of this highly pathogenic ESBL-producing *E. coli* lineage by conducting a phylogenetic comparison between Swedish and international isolates. We found, in accordance with our hypothesis, that several introductions from the international lineage have shaped the Swedish ST131 population. Tight genetic relationships between isolates in clonal clusters makes it difficult to perform outbreak investigations with ST131. In addition, we identified highly conserved plasmids in all clusters with Swedish isolates even though they had been separated for several years indicating a strong co-evolution of plasmids in some ST131 lineages.

Taken together our studies show that although there is a high prevalence of ESBL/pAmpC-producing *E. coli*, particularly in poultry and chicken meat products, the major source for ESBL/pAmpC producing *E. coli* causing human infections is humans to human transmission. Although we do not see a great contribution from the animal and food sector in Sweden it could change in the future if an epidemiological shift to more human pathogenic strains take place in e.g. poultry. This is why a multi-sectorial approach to reduce the levels of ESBL/pAmpC-producing *E. coli* in all sectors is needed.

Of particular interest is the highly pathogenic *E. coli* lineage ST131 that is responsible for a large proportion of infection with ESBL/pAmpC-producing *E. coli.* Carriers of ST131 could therefore be at greater risk of getting an infection and there might be incentive for considering them as high risk carriers. The high clinical relevance of ESBL-producing *E. coli* ST131 prompts further monitoring since this lineage has large potential to accumulate resistance to last resort drugs such as carbapenems and colistin.

LIST OF SCIENTIFIC PAPERS

I. Börjesson S*, **Ny S***, Egervärn M, Bergström J, Rosengren A, Englund S, Löfmark S, Byfors S. Limited Dissemination of Extended-Spectrum β-Lactamase- and Plasmid-Encoded AmpC-Producing *Escherichia coli* from Food and Farm Animals, Sweden. *Emerging infectious diseases* **22**, 634-640, doi:10.3201/eid2204.151142 (2016). **shared first author*

II. **Ny S,** Löfmark S, Börjesson S, Englund S, Ringman M, Bergström J, Naucler P, Giske CG, Byfors S. Community carriage of ESBL-producing *Escherichia coli* is associated with strains of low pathogenicity: a Swedish nationwide study. *The Journal of antimicrobial chemotherapy* **72**, 582-588, doi:10.1093/jac/dkw419 (2017).

III. **Ny S,** Kozlov R, Dumpis U, Edquist P, Gröndahl-Yli-Hannuksela K, Kling A-M, Lis DO, Lübbert C, Pomorska-Wesołowska M, Palagin I, Vilde A, Vuopio J, Walter J, Tegmark Wisell K, NoDARS ESBL-carrier working group. Large variation in ESBL-producing *Escherichia coli* carriers in six European countries including Russia. *European Journal of Clinical Microbiology & Infectious Diseases*, doi:10.1007/s10096-018-3382-8 (2018).

IV. **Ny S**, Sandegren L, Salemi M, Giske CG. Genome and plasmid diversity of ESBL-producing *Escherichia coli* ST131 – tracking phylogenetic trajectories with Bayesian inference. Manuscript

Papers not included in the thesis

I. Atterby C, Börjesson S, **Ny S**, Järhult JD, Byfors S, Bonnedahl J. ESBL-producing *Escherichia coli* in Swedish gulls-A case of environmental pollution from humans? *PLoS One* **12**, 1-13, doi:10.1371/journal.pone.0190380 (2017).

II. **Ny S**, Edquist P, Dumpis U, Gröndahl-Yli-Hannuksela K, Hermes J, Kling A-M, Klingeberg A, Kozlov R, Källman O, Lis D, Pomorska-Wesołowska M, Saule M, Tegmark Wisell K, Vuopio J, Palagin I , NoDARS UTI study group; Antibiotic resistance of *Escherichia coli* from outpatient urinary tract infection in women in six European countries including Russia. *Journal of Global Antimicrobial Resistance*, doi:10.1016/j.jgar.2018.11.004 (2018).

CONTENTS

LIST OF ABBREVIATIONS

pAmpC	Plasmid encoded AmpC
BEAST	Bayesian Evolutionary Analysis Sampling Trees
BSI	Bloodstream infection
CDC	Center for Disease Control and Prevention (USA)
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ESBL	Extended-Spectrum β-Lactamase
KPC	*Klebsiella pneumoniae* carbapenemase
MLST	Multi Locus Sequence Types
MIC	Minimal Inhibitory Concentration
MRCA	Most Recent Common Ancestor
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NDM	New-Delhi Metallo-β-lactamase
NGS	Next Generation Sequencing
UTI	Urinary Tract Infection
WGS	Whole Genome Sequencing

PREAMBLE

"Nothing in Biology Makes Sense Except in the Light of Evolution" is a 1973 essay by the evolutionary biologist Theodosius Dobzhansky[1]. Evolution theory is a fundamental concept in order to understand how antibiotic resistance arises and spreads. Yet today, 160 years after the publication of Charles Darwin's "Origin of Species", we are struggling to manage the effects of the natural selection process we have induced by using antibiotics. Our antibiotic use is actively changing the population structure of bacterial communities in and on our bodies as well as in the environment around us. As we move in an increasingly connected world, visualized by the global airline routes on the front page, antibiotic resistant bacteria travel with us.

Already in 1945, the "father of penicillin", Sir Alexander Fleming, warned about antibiotic resistance. But after the Second World War we entered the "golden age of antibiotic discovery" and between 1950 and 1970 most classes of antibiotics still used today were discovered[2,3]. The abundance of new antibiotics together with the good treatment efficacy made us pay little attention to the gathering evidence that antibiotic resistance was becoming a problem.

Today we are dependent on antibiotics – not only for the treatment of primary bacterial pathogenic infections such as *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae* – but also for stopping infections related to many fundamental medical procedures such as surgery, cancer treatment and neonatal care. In addition, many countries have created animal production systems that are dependent on antibiotics for the animals to grow up fast and healthy.

Antibiotic resistance is not a problem that can be solved, as long as we use antibiotics we will continue to exhort a selection pressure which will in the end lead to resistance. However, the way we currently use antibiotics must change if we want to continue using them in the future.

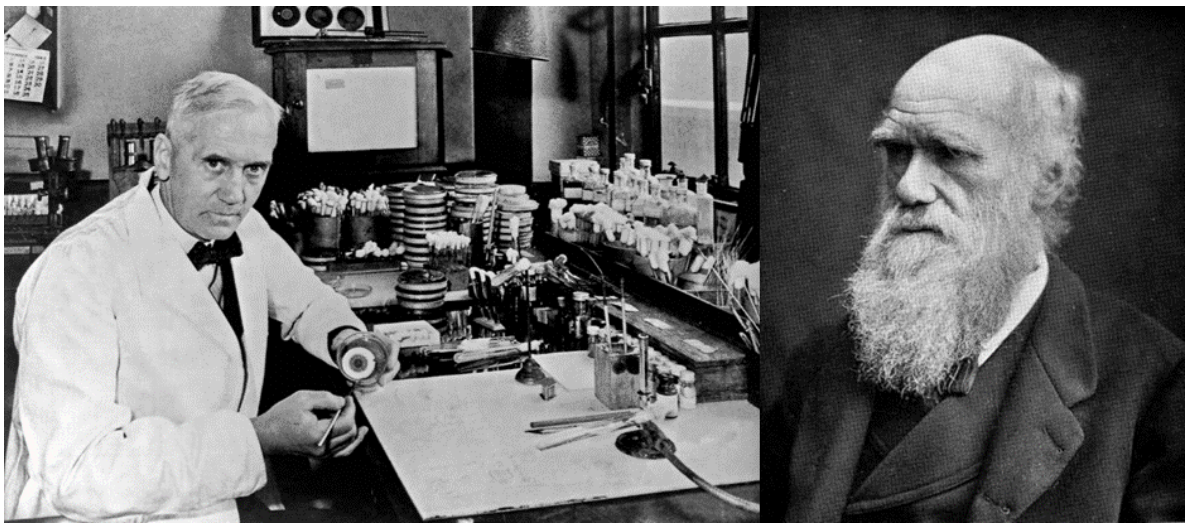

Figure 1. Sir Alexander Fleming and Charles Darwin. Photos by Davies Keystone and Lock and Whitfield.

1 INTRODUCTION

1.1 WHAT IS ANTIBIOTIC RESISTANCE?

Antibiotic resistance is defined as the ability of a bacterium to resist an antibiotic that the majority of that bacterial species would be otherwise susceptible to. Natural selection will favour bacteria with mutations which give them resistance to the antibiotic they are subjected to. In simple terms, during antibiotic treatment, the susceptible bacteria are killed leaving the resistant bacterial population to expand. Antibiotic resistance can be gained through different evolutionary pathways, but is brought on by the use of antibiotics which create a selection pressure on the bacterial population.

During the years, we have successfully developed treatment strategies based on plasma concentration needed to cure the patient and the Minimal Inhibitory Concentration (MIC) of an antibiotic. MIC refers to the dose needed to inhibit further growth of the specific bacterium causing the infection. The MIC values are determined and measured in laboratory conditions that might not be an ideal representation of *in vivo* conditions. We know today that bacterial in vivo populations are far from homogenous, for example they form biofilms which allows them to survive antibiotics better and some bacterial cells can be persisters, which means that they survive antibiotic by temporary growth arrest[4,5]. Recent evidence also suggests that heteroresistance, i.e. "the presence of a resistant subpopulation in a main population of susceptible cells", can play a role in treatment failures of an, according to MIC, otherwise susceptible population[6].

1.1.1 Evolution of resistance in real time

To demonstrate bacterial evolution, from susceptible to resistant against antibiotics, in real time researchers created a microbial evolution and growth arena (MEGA) plate with increasing concentration of antibiotics towards the middle of the plate, Figure 2. The top layer of the plate allowed bacteria to move and grow into a higher concentration of antibiotics, and gain more nutrients, if they developed resistance. In addition to creating innovative time-lapse movies of evolution they could also study mechanisms such as adaptation, fitness, mutation rates and expansion patterns showing that evolution is not always driven by the most resistant and fittest mutants but rather by the mediocre specimens. It took the sensitive *Escherichia coli* (*E. coli*) strain 12 days to reach a 3,000-fold phenotypic resistance increase from the initial wild-type concentration of the antibiotic[7]. Experiments like this shows us how unchallenging it is for *E. coli* to develop high levels of resistance, after all they have had thousands of years to practice.

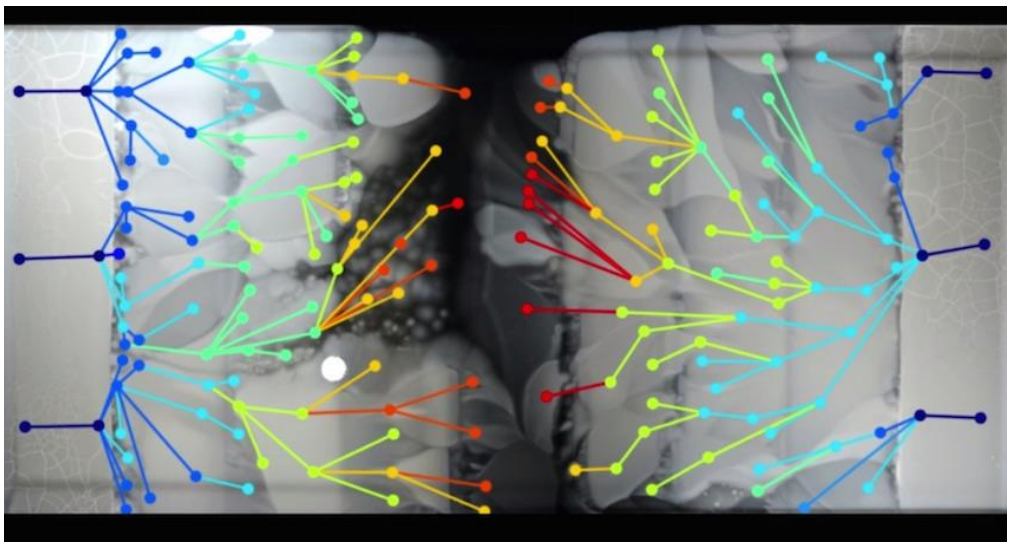

Figure 2. The microbial evolution and growth arena plate with inwards increasing levels of trimethoprim 12 days after inoculation with a sensitive *E. coli* strain (dark blue). The dots and colours represent pheno- and genotypically different clonal lineages. Rights to reprint Elsevier[7].

1.2 ENVIRONMENTAL RESISTANCE

Antibiotic resistance is a natural occurring phenomenon and resistance genes are ubiquitous in the environment. Many of our most common antibiotics, such as penicillin, are naturally produced by soil dwelling fungi and bacteria to compete over resources with other soil microorganism. To fight back bacteria developed their own defence – antibiotic resistance.

A recent metagenomic study on 189 soil samples representing terrestrial regions from all over the world revealed a correlation between abundance of fungi and high content of antibiotic resistance genes. The researchers also saw that in soils with high content of antibiotic resistance genes the phylogenetic diversity of the bacterial populations was lower[8].

Naturally occurring resistance creates a problem when the genes coding for the resistance transfers to pathogenic bacteria that cause human or animal infections. Some of the most widely spread and problematic resistance genes, such as $bla_{\text{CTX-M}}$ encoding resistance to third generation cephalosporins, are believed to originate from soil bacteria of the *Kluyvera* spp. [9,10]. By extension, this means that all naturally occurring antibiotics likely already have resistance genes in the natural habitat of the organism that produces the antibiotic. The fact is that resistance has developed to all major classes of antibiotics ever discovered, which is slightly discouraging[11]. In addition, the time between when the antibiotic is taken into clinical practice to when resistance emerges has been less than 10 years for several antibiotics[12,13].

In later years antibiotics as well as antibiotic resistance genes have also come to be considered as environmental pollutants[14]. Overuse and misuse during the last half century in both the human and agricultural sector have created an abundance antibiotic substances which can exert selection pressure when released into the environment[15]. There is a great concern around what effect antibiotics released into the environment will have on microbial communities and their ability to perform fundamental functions[16,17].
The presence of antibiotics also has an effect on the microbial communities on our body and

in later years research around our microbiome and how it is affected by antibiotics has increased rapidly.

1.3 MECHANISMS OF ACTION FOR ANTIBIOTICS AND ANTIBIOTIC RESISTANCE

1.3.1 Mechanisms of action for antibiotics

Antibiotics are compounds that bind to specific targets in the bacterial cell. In order for an antibiotic to be efficient it needs to target an essential bacterial cell function which will lead to growth arrest (bacteriostatic) or cell death (bactericidal). Antibiotic classes have different targets within the cell, some displayed in Figure 3A.

β-lactam antibiotics, including cephalosporins, attacks the cell wall synthesis by binding to penicillin-binding proteins and thereby inhibit the formation of the bacterial peptidoglycan layer[18]. This leads to growth arrest because of a weaker cell wall which often results in cell death. Other important cell targets for antibiotics are the DNA/RNA synthesis, protein synthesis and the folate metabolism, Figure 3A. For example fluoroquinolone antibiotics, inhibits DNA replication by binding to DNA gyrase and topoisomerase IV, which are involved in the uncoiling of DNA before replication, and induce double-stand breaks in front of the DNA polymerase[19]. Aminoglycosides inhibit protein synthesis by binding to the 30S subunit which causes misreading of the mRNA and/or early termination of protein synthesis[20].

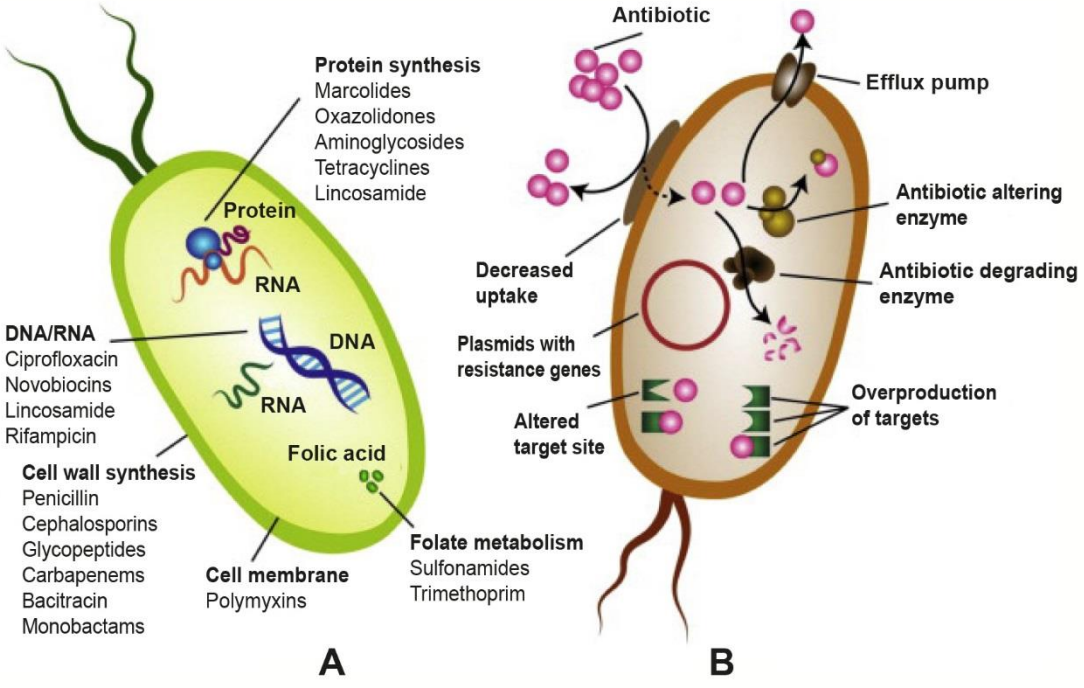

Figure 3. A. Antibiotics targets different essential functions in the bacterial cell. **B.** Mechanisms for intrinsic and acquired resistance. Rights to reprint from Elsevier[21].

1.3.2 Mechanisms of antibiotic resistance

Antibiotic resistance can be intrinsic or acquired. Intrinsic resistance is the bacterial capability to resist a specific antibiotic naturally. It can be because the antibiotic lack the ability to enter through the cell wall or that the bacterium simply does not have the target receptor that the antibiotic uses to exercise its effect. For example, Gram-negative bacteria are intrinsically more resistant to several antibiotics since its outer membrane makes it less permeable[22-24].

Acquired resistance is when a bacterium develop or acquire resistance through gene mutation or horizontal gene transfer. Mutants can then be selected by antibiotics. Acquired resistance can be achieved by several mechanisms, some displayed in Figure 3B, which can be divided into three main categories; i) decreased uptake and increased efflux of antibiotics from the bacterial cell, ii) target modification by mutation and iii) modification or hydrolysis of the antibiotic which makes it inactive. In Table 1 some common antibiotics and resistance mechanisms have been summarized.

Sometimes intrinsic resistance can become acquired resistance, one example is multidrug resistance (MDR) efflux pumps, an example of resistance due to increased efflux of drug. All bacteria carry genes encoding efflux pumps, with different substance spectrums, on their chromosomes which is classically considered an intrinsic resistance mechanism. However, some efflux pumps have moved onto plasmids making them transmissible between bacteria. For example, an MDR efflux pump was found on a plasmid co-harbouring the carbapenemase NDM-1 (New-Delhi Metallo-β-lactamase) in a *Citrobacter freundii* strain[25]. Transferrable MDR efflux pumps like these have a large potential to become clinically important since they can transmit to pathogenic bacteria and cause resistance to several antibiotics at once.

Modification of target by mutation means that the bacterial target is modified by e.g. point mutation or uptake of a different but homologous allele by recombination. On example is methicillin resistance in Methicillin-Resistant *Staphylococcus aureus* (MRSA) which is gained by uptake of a genetic element, including the *mecA* gene. The *mecA* gene produces an enzyme which makes the synthesis of the peptidoglycan layer possible even during exposure to penicillins[22,26].

Inactivation by hydrolysis consists of thousands of different enzymes and is clinically the most important resistance mechanism. It includes a large group of different β-lactamases which hydrolyse a range of important antibiotics like penicillins, cephalosporins and carbapenems. The mechanism of action of β-lactamases towards penicillin is hydrolysis of the β-lactam ring, which is the structure on the penicillin responsible for the drug activity. Bacteria that are resistant to penicillin because of β-lactamase production can be treated by 3rd generation cephalosporins. However the Extended-Spectrum β-Lactamases (ESBL) can also hydrolyse 3rd generation cephalosporins[22,27].

Table 1. Mechanisms of resistance to different classes of antibiotics. Table adapted from Kumar et al 2013 with permission from Nexus Academic publishers[28]

Antimicrobial class	Mechanism of Resistance	Specific means to achieve resistance	Examples
β–lactams	Degradation of antibiotic	Destruction of lactam ring by β-lactamases making it incapable to bind with penicillin binding protein (PBPs).	Resistance to penicillins, cephalosporins and carbapenems.
	Altered target	Mutational changes PBPs	Methicillin and oxacillin resistant staphylococci.
	Decreased uptake	Decreased porin channel formation.	Imipenem resistance of *K. pneumoniae*, *E. aerogenes*, and *P. aeruginosa.*
Aminoglycosides	Enzymatic modification	Acetylation	Aminoglycoside resistance in Gram-negative and positive bacteria
	Decreased uptake	Change in number or character of porin channels.	Aminoglycoside resistance in Gram-negative bacteria.
	Altered target	Modification of ribosomal proteins or 16S rRNA.	Streptomycin resistant *Mycobacterium* spp.
fluoroquinolones	Decreased uptake and increased excretion	Alterations in the outer membrane diminishes uptake of drug. Activation of efflux pumps to excrete drug.	Fluoroquinolones resistant Gram-negative bacteria and staphylo-cocci.
	Altered target	Changes in DNA gyrase.	Fluoroquinolone resistant Gram negative and positive bacteria
Glycopeptides	Altered target	Alteration in cell wall precursor which decreases the binding of vancomycin and teicoplanin.	Vancomycin resistant enterococci.

1.3.3 Transferrable resistance – with plasmids in focus

Once genetic antibiotic resistance has developed in one bacterial strain, it can transfer to other bacteria via horizontal gene transfer, which can take place between bacteria of the same or different species. There are three mechanisms of horizontal gene transfer which are 1) conjugation, 2) transduction and 3) transformation, Figure 4[29].

Plasmids are circular extra chromosomal DNA elements which replicate independently in the cytoplasm of the bacterium. They often contain non-essential genes that sometimes gives the bacterium a selection advantage, like antibiotic resistance genes. Plasmids are inherited vertically, from mother to daughter cell, at replication. Many plasmids also have the ability to transmit horizontally between bacterial cells and are called conjugative plasmids Figure 4. To ensure stable inheritance many plasmids contain toxin-antitoxin addiction systems which kills the new daughter cell if it does not contain the plasmid[30]. Because plasmids are self-replicating units that have an individual evolutionary history some scientist consider them living organisms[31].

One of the main transmission routes for ESBL genes is via conjugative plasmids. The plasmids can be characterised by separation into different incompatibility groups depending on differences in replicon types present on the plasmids. The most common incompatibility groups in connection to ESBL/pAmpC genes are IncF, IncI1, IncK and IncN[32,33]. The success of the global epidemic *E. coli* clone ST131 is tightly linked to conjugative IncF plasmids. Transduction via bacteriophages has also been shown to have an impact on the diversity of the ST131 genome[34].

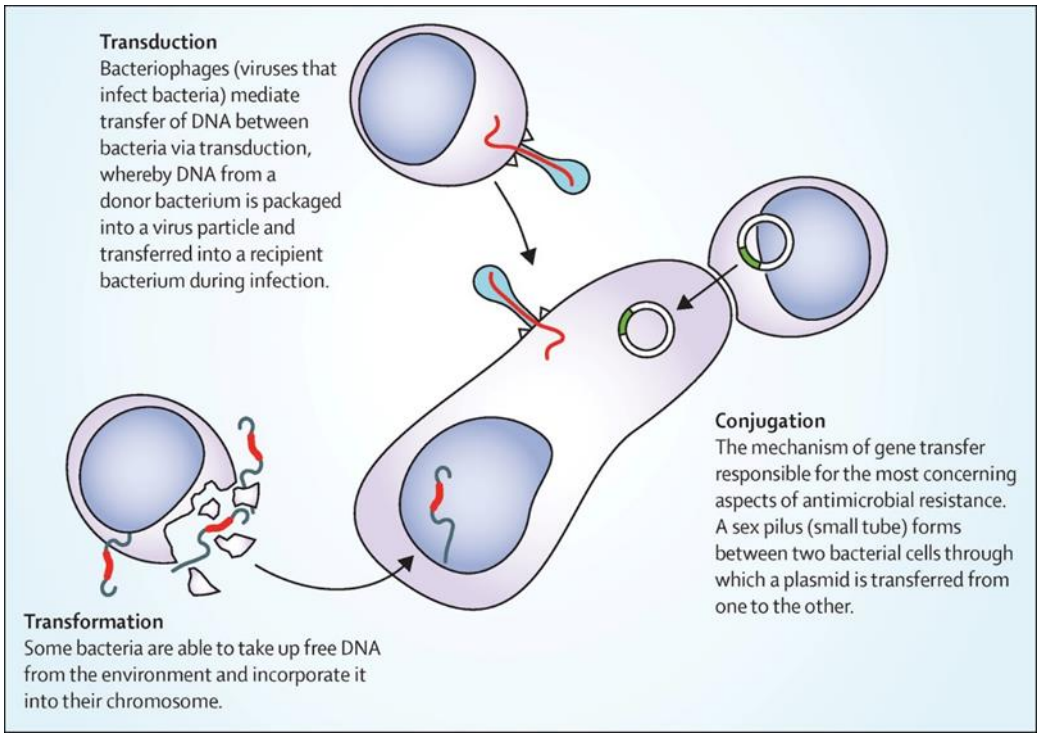

Figure 4. Mechanisms for horizontal transfer of genetic material in bacteria. Rights to reprint via Elsevier[35].

1.4 WHAT ARE ESBLs?

ESBLs are enzymes, produced by bacteria, which have the ability to hydrolyse extended-spectrum cephalosporin antibiotics (as well as penicillins). In the classical definition of ESBL the gene also has to be transmissible horizontally between bacteria on some sort of mobile element like a plasmid[36,37]. ESBLs were first detected in a clinical isolate of *Klebsiella ozaenae* in the beginning of the 1980s[38]. A rapid development and spread of several different classes of ESBLs followed[39,40].

There are different ways of classifying ESBL and β-lactamases. One commonly used system is Ambler classification that divides β-lactamases based on the amino acid sequence, and what mechanism that is used for hydrolysis (serine- vs metalloenzymes), into four categories A, B, C and D[41-44]. In Sweden we tend to use the definition $ESBL_A$ (enzymes like CTX-M, TEM, SHV which are inhibited by clavulanic acid), $ESBL_M$ (a miscellaneous category including enzymes like plasmid mediated AmpC β-lactamases) and $ESBL_{CARBA}$ (enzymes

giving carbapenem resistance like NDM and KPC)[36,37]. The focus of this thesis is on $ESBL_A$ and $ESBL_M$ expressed as ESBL/pAmpC for clarity.

The most common ESBL-enzymes worldwide are CTX-M, TEM, SHV and OXA[45]. The CTX-M are both the largest group and the most prevalent enzymes in human bacterial pathogens. Since 2009 a variant called CTX-M-15 has dominated but also CTX-M-14, CTX-M-1 and CTX-M-27 are commonly identified[46].

1.4.1 Clinically important bacterial species associated with ESBL/pAmpC

A plenitude of different bacterial species can carry ESBL/pAmpC. However, the most important species for human clinical infections is in the Enterobacterales family. In addition, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are sometimes identified with ESBL. These two species also feature high intrinsic resistance to many antibiotics making them clinically hard to treat and therefore of high public health interest. In Sweden only ESBL identified in Enterobacteriaceae are notifiable according to the communicable disease act. This thesis is focused on ESBL/pAmpC-producing *E. coli* because of their diverse ecology and great zoonotic potential. ESBL/pAmpC-producing *Klebsiella pneumoniae* is also an important pathogen but mainly associated with nosocomial transmission. In two of the papers included in this thesis we also investigated community carriage of ESBL-producing *K. pneumoniae* but positive carriers were only identified in paper III. When investigating transmission routes for ESBLs connected to foods and community carriage, *E. coli* will have the most important role as vehicle for expansive dissemination.

1.4.2 *E. coli* biology and typing

Already back in 1893 a Danish veterinarian hypothesised that some *E. coli* species were pathogenic while others were not, contrary to Koch's postulate (1884) that bacterial species are either pathogenic or non-pathogenic[47].
Today *E. coli* can be divided into three separate categories depending on their level of pathogenicity[34,48]. The first is commensals that most of the time lack virulence factors and are therefore not pathogenic. In many humans these are part of the natural gut microbiota. The second is extra-intestinal pathogenic *E. coli* (ExPEC) which can infect humans and cause e.g. urinary tract infections (UTI) and/or bloodstream infections (BSI). These can be part of the normal human gut microbiota but also possess virulence factors necessary to cause infections outside of the intestinal tract. The third one is pathogenic intestinal *E. coli* which are not normally present in healthy humans. These have been divided into six different pathotypes, one of them including EHEC (Enterohemorrhagic *E. coli*)[34].

Pathogenic *E. coli* has a wide range of virulence factors which allows them to attach and interact with mucosal membranes, invade and later multiply in our bodies. To attach to

surfaces *E. coli* uses different types of adhesins such as fimbriae which are tail-like structures on the outside of the bacterium. Fimbriae, and other adhesins, help *E. coli* to colonise the intestine but also to form colony-like structures by attaching to each other[49]. Some *E. coli* also produce toxins the most famous being the shiga toxin originating from *Shigella dysenteriae* but also produced by STEC (Shiga Toxin producing *E. coli*).

The *E. coli* genome consists of one chromosome around 5 Mb in size and often one or several plasmids. The content of the genome is extremely variable depending on which type of *E. coli* you are looking at.

Attempts have been made to characterize the pan- and the core genome of *E. coli*. The fundamental behaviour of the pan- and core genome are that as you add more strains one will increase (pan) while the other decreases (core). One study using whole genome sequences from 61 different *E. coli* (including *Shigella* spp) found that the pan-genome consisted of around 15,741 gene families while the core genome had 993 gene families. This means that on average a minority of gene families, only around 6%, are present in every *E. coli* which makes the accessory genome vast in comparison[50]. With this information at hand we have to question the set-up and usefulness of one single core genome as a typing tool for *E. coli.* Typing techniques of *E. coli* strains that causes infections have since around 10 years back been centered on Multi Locus Sequence Typing (MLST). In this method 7 housekeeping genes are sequenced, and each unique allelic sequence is given a distinct number. Together the numbers from the allelic sequences are organized in sequence types (STs), where each unique allelic barcode translates to a unique ST[51]. In recent years, due to the increasing availability of WGS-data, the classical MLST scheme has been expanded to core genome MLST (cgMLST) which follows the same principle. The idea with cgMLST is to define a set of genes that are present in all bacteria of the species of interest. Also schemes for whole genome MLST (wgMLST) including both core and accessory genes have been developed for several bacteria[52,53]. Both cgMLST and wgMLST can and are widely used as the basis for SNP-calling to generate SNP-alignments to use for phylogenetic analysis.

1.5 ESBL/pAmpC-PRODUCING *E. COLI* IN HUMANS

The epidemiology of ESBL/pAmpC-producing *E. coli* in humans is multi-sectorial and fast changing. Several possible transmission routes in combination with different transmission entities (i: genes, ii: plasmid with genes or iii: bacteria with genes and/or plasmids) makes it a challenging task to understand the bigger picture. Figure 5 represents a simplified example of how ESBL/pAmpC-producing *E. coli* disseminates. In the centre of the story we find human community carriers that work as transmission mediators for ESBL/pAmpC-producing *E. coli*, from different sources, to clinical infections in humans. The transmission is faecal-oral and you can become a carrier by consuming bacteria from contaminated food or by interaction with contaminated environments or other carriers. ESBL/pAmpC-producing *E. coli* disseminate both via clonal transmission of successful bacterial lineages and via plasmids

between bacteria. When we consume antibiotics, we select for the bacteria harbouring the ESBL/pAmpC genes in our gut microbiome. Far from all ESBL/pAmpC-producing *E. coli* carriers get infections from their unwanted passengers, which makes most carriers non-symptomatic. This adds a layer of complexity since the transmission between relatively healthy community carriers takes place in silence.

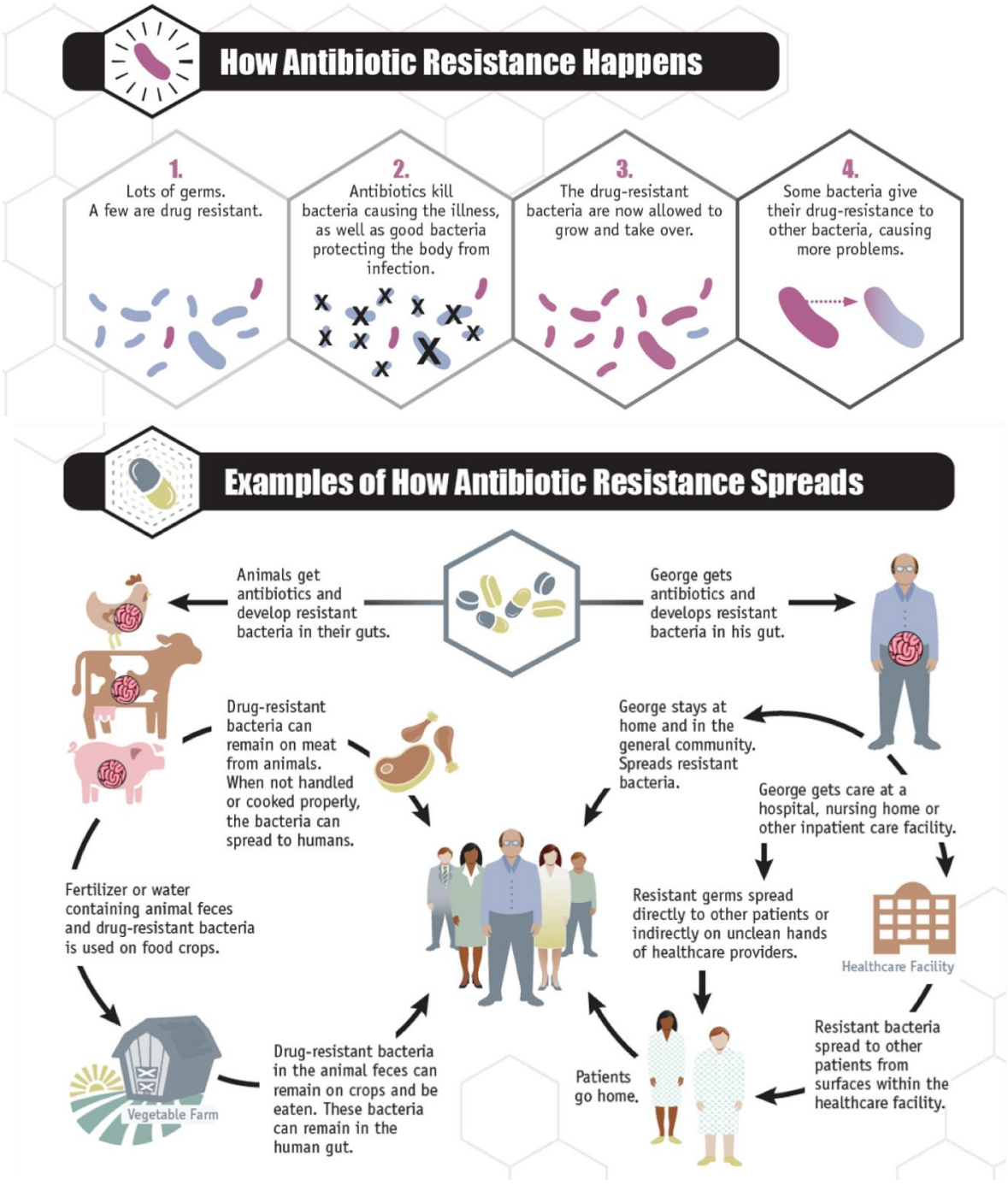

Figure 5. Schematic drawing representing the quintessential examples of how ESBL/pAmpC-producing *E. coli* are established and spreads among humans and in our environment. Right to reprint, CDC Stacks[12].

1.5.1 Risk behaviours connected to community carriage of ESBL/pAmpC-producing *E. coli*

Intestinal carriage can be detected if a patient is screened e.g. upon admittance to hospital or in specific screening studies. There are no regular surveillance systems that monitor intestinal carriage of ESBL/pAmpC producing *E. coli* in the community in Sweden. However, several studies have been done especially in connection to investigating foreign travel as a risk factor for becoming a carrier. The results suggest that travel to high prevalence countries, especially in northern Africa and Southeast Asia is a risk factor for becoming a carrier[54-61].

It is clear that travels contribute to the prevalence of community carriers in Sweden, however it is difficult to estimate how this population effects the overall prevalence in society since other factors such as health-care association, food intake and antibiotic use contributes to carrier prevalence. In paper II, we investigated the risk factors connected to carriage on a community level as opposed to only studying travellers.

International surveillance initiatives are lacking when it comes to community carriage but local studies on carriers exist in some countries[59]. An increasing trend of carriage of ESBL/pAmpC producing *E. coli* has been described since the beginning of the 2000s and the prevalence ranges between a few percent and up to 60-70% for populations investigated in Thailand and Egypt[59,62,63]. A review and calculation on pooled prevalence based on studies reporting faecal carriage concluded an average worldwide carrier prevalence of 14% with an annual increase of 5.4% between 1991 and 2014[64]. Taking this evidence into account one can only conclude that the carriage prevalence of ESBL/pAmpC-producing *E. coli* is significant and that it likely contributes to community acquired infections that are hard to treat and could lead to treatment failures. This makes it urgent to better understand the epidemiology of ESBL/pAmpC-producing *E. coli* in society so that prevention measures to curb the dissemination can be put into place. To investigate the epidemiology of ESBL/pAmpC-producing *E. coli* in Northern Europe we combined efforts with five other countries and conducted a prevalence study on carriers in Finland, Latvia, Germany, Poland, Russia and Sweden, Paper III.

There has been much debate in recent years regarding the prevalence of ESBL/pAmpC -producing *E. coli*, including carbapenemase-producing *E. coli* in refugees seeking asylum. The debate both concerns the dissemination in the community but also how to apply screening strategies towards this group. People seeking asylum in Sweden often originate from countries with suspected high prevalence of ESBL/pAmpC-producing *E. coli* in the community, like Syria, Afghanistan, Iraq, Somalia and Eritrea. However, because studies from these countries are limited it is impossible to make evidence-based conclusions. In a recent study from Germany, including 1,544 asylum seekers, 294 i.e. 19% tested positive for ESBL but zero carbapenemase resistant isolates was identified. The most common ESBL genes identified were $bla_{\text{CTX-M-15}}$ (79%) and $bla_{\text{CTX-M-27}}$ (10%) and ST131 accounted for 24% of MLSTs among carriers[65]. As a comparison approximately 20-30% of tourists visiting

Southeast Asia are positive for ESBL when they return from their trip[54,55,61]. Annually about 200,000 Swedes visit Thailand[66].

1.5.2 Trends in surveillance of invasive infections caused by ESBL/pAmpC producing *E. coli*

On a European level EARS-Net monitors the prevalence of antibiotic resistance to several important antibiotics, in isolates from BSI[67]. Since the surveillance began in 2000, in total 31 countries have joined and are reporting data each year. The monitoring uses phenotypic resistance and the proxy for estimating ESBL/pAmpC is resistance to 3rd generation cephalosporins. In Figure 6 a summary of the resistance to 3rd generation cephalosporins in *E. coli* causing BSI reported by members since 2005 shows a steady increase over time. The monitoring is sensitive to cultivation numbers, which forms the denominator, and varies greatly between countries because of the use of different clinical indications on when to take a blood culture. If only severe cases are cultured the probability of the isolate causing the infection being resistant increases, which inflates the proportion of resistance isolates. This can be seen in Figure 6 where the proportion of resistant isolates varies greatly from one year to another for some countries. In Sweden, where blood cultures are taken generously, the estimated ESBL/pAmpC proportion in *E. coli* BSI has increased from 1.7% in 2005 to 7.7% in 2017[68-70]. Central Asia and Eastern Europe has a similar network called CAESAR[71].

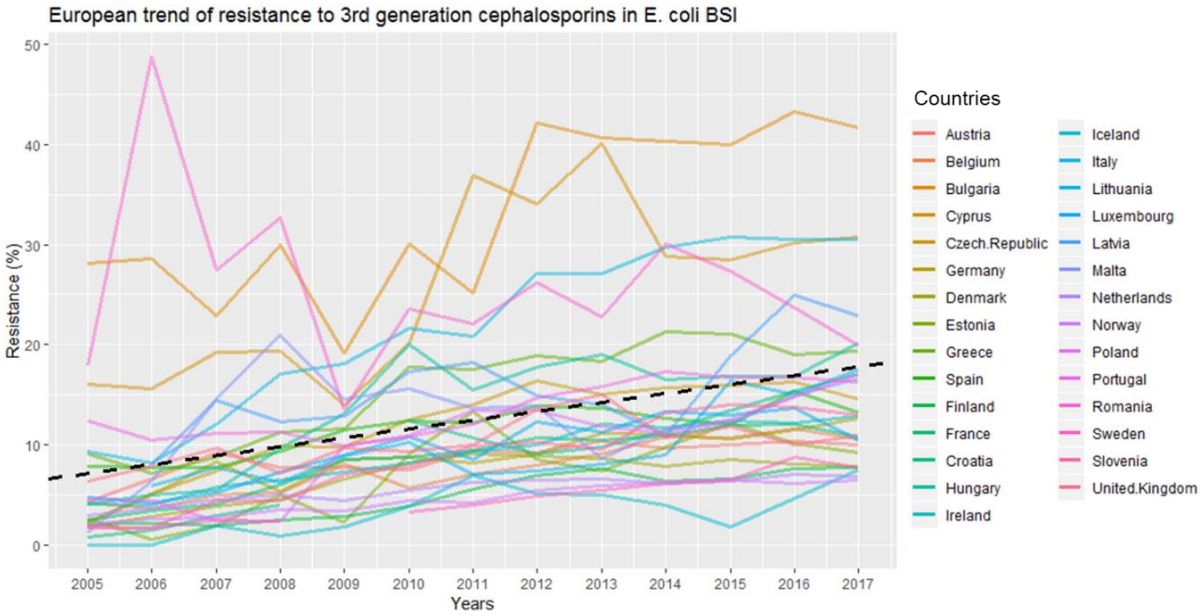

Figure 6. General trend shows an increase in resistance to 3rd generation cephalosporins in European countries during the last 12 years.

According to Swedish recommendations cephalosporins are part of the first line treatment for BSI, and it is therefore important to monitor trends since a high share of resistance can lead to

treatment failures[72]. One important risk factor for having a BSI with ESBL-producing Enterobacteriaceae is previous ESBL-positive culture (carriage and/or infection)[73-75]. The number of people at risk for ESBL-BSI could therefore increase if community carriage increases[75].

1.6 ESBL IN RESISTANCE EPIDEMICS

Sometimes an antibiotic resistance gene end up in a genetic environment, like close to an active transposon or get moved onto a successful plasmid, which allows it to spread fast to several different bacteria. Some well-known examples of this are the *bla*$_{\text{CTX-M-15}}$ association with an active *ISEcp1* transposase and the colistin resistance gene *mcr-1* which has disseminated to a range of plasmids via its association to the *ISApl1* transposon[76-78]. When a successful resistance gene end up in a bacterial host with favourable traits the combination can make the bacteria very successful because of its ability for rapid dissemination, colonisation and persistence. These bacteria are known as high-risk clones. The high-risk clones are responsible for epidemic spread leading to endemic situations in some settings.

One example is the nosocomial spread of KPC-producing *K. pneumoniae* ST258 which has been ongoing since the beginning of 2000[79]. Which exact traits that defines a high-risk clone are under debate but they are generally described as a combination of virulence factors and multidrug-resistance[34,77,79,80].

1.6.1 *E. coli* ST131 and its subclones

In 2008 a new *E. coli* sequence type called ST131 was discovered in clinical isolates at several places simultaneously[80-83]. A few years later it was, and still is, the most common sequence type in human clinical infections with ESBL-producing *E. coli*[84].

ST131 is a lineage of *E. coli* that can cause extra-intestinal infections (ExPEC) and almost exclusively belong to phylogenetic group B2[80]. The lineage is mainly connected to the gene *bla*$_{\text{CTX-M-15}}$ and plasmids with replicon type IncF and is often multidrug-resistant. Almost all clinical strains of ST131 are also resistant to fluoroquinolones which means that the widespread use of this substance, for treating common infections such as UTIs, has likely contributed to selection for this sequence type[76,77,81,85,86]. In Sweden the share of ST131 has been around 30-40% in UTIs caused by ESBL-producing *E. coli* in national surveillance since 2007[87]. In manuscript IV we performed a modulation of the ST131 emergence which supports the observation of a stable proportion of ST131 in infections since 2005.

Two studies suggested in 2013-2014 that the spread of ST131 was due to the dissemination of one single highly pathogenic sub-clone called H30-Rx or C2 (referred to as C2)[88,89]. The evidence presented was based on Whole Genome Sequencing (WGS) (Illumina) and the construction of maximum likelihood and maximum parsimony phylogenies. The clade C2

was separated by few SNPs, and almost exclusively contained *bla*$_{\text{CTX-M-15}}$. The conclusion was that instead of several independent acquisition events of plasmids of IncF replicon type containing the gene *bla*$_{\text{CTX-M-15}}$ the emergence was connected to a single clone. The calculations regarding when this emergence took place differed somewhat with one study suggesting the mid-1980s[88] and the other before 2000[89].

Further investigations of the evolutionary history of this clone has been done with a time-scale phylogeny using BEAST (Bayesian Evolutionary Analysis Sampling Trees). The authors concluded that the C2 clone had emerged around 1987 and that the geospatial patterns were weak[86,90]. The mutation rate was determined to around 1 SNP per year per genome. In the analysis, a strict clock model was assumed together with constant population size[90]. Another modulation, using the same method, gave similar results but used a different model for the mode of mutations as well as the prior tree model the data was fitted to. However, the overall result regarding tree topology, clade separation and approximate time of emergence were similar between the studies[86]. None of the global phylogenetic studies have included ST131 from Sweden. In manuscript IV we investigated the introduction patterns of ST131 producing ESBL in Sweden and how the Swedish isolates relate to the global epidemiology.

1.7 ESBL AND pAmpC IN FOODS AND FARM ANIMALS

Total antibiotic use and resistance emergence are closely linked to the use of antibiotics in agriculture. By overuse and misuse in the agricultural sector we are accelerating the evolution and emergence of resistance. Tackling antibiotic resistance therefore becomes a "One Health" project since the majority of antibiotic usage globally are in agriculture and animal husbandry. Actions are taken, for example in the United States, where use of antimicrobials as growth promoters to healthy animals is legal, new legislation has decreased the use of clinically important antibiotics usage to farm animals by 43% between 2015 and 2017[91]. However a study estimated that the antimicrobial consumption in livestock production will increase by 67% between 2010 and 2030. About a third of the increase is estimated to be due to changing production in middle-income countries since the demand for meat is on the rise[92].

ESBL/pAmpC-producing *E. coli* has a history of presence in farm animals and a specifically intimate relationship with poultry production[93-96]. However, reports of ESBL/pAmpC in pig and cattle farms show that the problem is far from isolated to poultry production[97-99].
The poultry production is organized in a pyramidal structure where few individuals at the top of the pyramid (called pedigree and Great Grandparents Stock) gives rise to several generations before reaching the broiler level at the bottom, which are the chickens we consume. Studies have shown that transmission downward in the pyramid can take place[100-103]. Because of this structure it is very hard to get rid of the resistant bacteria once they have been introduced.

In late 2015 a study from China described plasmid-mediated colistin resistance for the first time which was found to have emerged from use of this substance in pig production for many years[104]. This was especially devastating (even if not unexpected) since colistin is nowadays often the last resort treatment option for carbapenem resistant Gram-negative bacilli.

In 2011 the European Food Safety Authority (EFSA) Panel on Biological Hazards released a scientific opinion on "the public health risks of bacterial strains producing Extended-Spectrum β-lactamases (ESBL) and/or pAmpC β-lactamases (pAmpC) in food and farm animals"[105]. The panel concluded that the most important genes for possible dissemination between animals and humans are *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{CMY}}$. They also concluded that although the prevalence of ESBL/pAmpC in farm animals is largely unknown it has been connected with the production pyramid in poultry and efforts should be taken on a European level to reduce the use of antibiotics in animal production. The panel further wrote that there is a possible risk for transmission of ESBL/pAmpC producing bacteria from farm animals (mainly poultry) to humans via the food chain[105].

High levels of ESBL/pAmpC-producing *E. coli* in poultry has been reported globally and from several European countries[106]. Prevalences often ranges from 40 to 100%[95,100,107]. The bacteria are present throughout the broiler production pyramid and disseminate despite the absence of selection pressure from antimicrobial usage[100,108,109].

In 2010 the Swedish National Veterinary Institute (SVA) discovered a 34% prevalence of ESBL/pAmpC-producing *E. coli* among Swedish broilers in the surveillance program (SVARM) despite very low antibiotic use in the sector. This indicated that imported breeding stock could be part of the problem[110]. The genes present on broilers in Sweden are mainly *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CMY-2}}$[69].

1.7.1 Transmission of ESBL/pAmpC from foods and farm animals to humans

One important question to answer is if transmission of ESBL/pAmpC-producing *E. coli* from farm animals via the food chain to humans occur. If the answer is yes, a follow-up question is; to what extent does this transmission contributes to disease in humans? That transmission likely can occur has been reported in several studies[111-113]. However, evidence suggests that the spread might be via plasmids and not clonal lineages and might vary depending on the local epidemiology in poultry[111,112]. In Sweden 44% of chicken filets are contaminated with ESBL/pAmpC producing *E. coli*, so we obviously consume these bacteria on a regular basis[114,115].

In 2011 a study from the Netherlands showed a large genetic overlap in ESBL genes from *E. coli* isolated from chicken meat, human faecal samples and human blood cultures and concluded that the increase in ESBL/pAmpC seen in humans could be due to transmission via the food chain[96]. Further analysis identified *E. coli* MLST ST10, the ESBL-gene *bla*$_{\text{CTX-M-1}}$ and plasmid replicon type IncI1 as the most common overlapping isolates between chicken

meat and human isolates[111]. This, and other studies, inspired a range of similar investigations, including our paper I, since the authors concluded that the prevalence in poultry could be the reason for the increase seen in human infections.

Several studies have shown that farmers have a higher risk of being colonised with ESBL/pAmpC-producing *E. coli*[97-99,116]. This is not surprising considering the high prevalence of ESBL/pAmpC in especially poultry. However, a recent study compared ESBL/pAmpC-producing *E. coli* carrier rates in poultry, farmers and non-farmers in rural Vietnam and found juxtapose results, here farmers had significantly lower carrier prevalence. In fact, the chickens had a much lower carrier prevalence, of 14.7%, compared to humans at 40.2% in average. In this setting the chicken production was non-intensive which indicates that the increased risk for becoming carrier as a farmer might only apply to farmers working in specific poultry production systems. On the other hand, carrier prevalence was altogether very high for humans, 31.9% for farmers compared to 49.5% in non-farmers, which might dilute the effect of the contribution from the chickens[117].

There is a great concern that one of the clinically most problematic *E. coli* lineages, ST131, will establish a reservoir in poultry because of its human pathogenicity and because it is often associated with ESBL. Unfortunately, there are already some indications that this is taking place. In 2012 researchers in the US collected and typed isolates, using NGS, from chicken meat and human Urinary Tract Infection (UTI) in a prospective study. They found that ST131 with a specific type of fimbriae, *fimH*22, was present in both chicken and the human clinical samples, accounting for 2% of the clinical infections[118]. All in all, 2% is not an overwhelming number but still an unwelcome contribution from chicken meat to human clinical infections. There are also case reports of the ST131/*fimH22* causing human BSI[119]. The *fimH22* allele is associated with clade B in the ST131 phylogeny which is not closely associated with ESBL-production[118]. In addition, ST131 was also detected on chicken meat in Italy in 2015[120].

In 2017 the European Centre for Disease Control, European Food Safety Authority and European Medicines Agency (ECDC/EFSA/EMA) released a report (JIACRA) where they used statistical modelling (Partial Least Squares Path Modelling) to determine which transmission routes that are the most important for the resistance seen in humans. The model included antibiotic resistance and antibiotic consumption in farm animals and humans and was performed for several different pathogens. The result showed that for ESBL-producing *E. coli* the main reason for antibiotic resistance in humans was antibiotic consumption in humans and not prevalence of resistance or consumption of antibiotics in animals. However, for *Salmonella* spp and *Campylobacter jejuni*, which are spread by clonal transmission, there were clear connections between antibiotic resistance in animals and humans[121]. Even though these results are modulation the results are interesting. However, the authors concluded that it remains to be elucidated how plasmid spread effects models like these.

One worry from the very successful dissemination of ESBL/pAmpC in humans and animals is that a similar spread with carbapenemase-producing bacteria will take place if prevention measures are not taken.

1.8 PHYLOGENETIC ANALYSIS

Mutations arise constantly and randomly across the bacterial genome when the DNA polymerase makes mistakes which are not corrected or wrongly corrected. This gives rise to changes like single nucleotide polymorphisms (SNPs). The SNPs can have several different effects on the protein being expressed but can also be silent, i.e. no phenotypic effect is seen because of the mutation. If the SNP gives rise to an amino acid change that may affect the ability of the bacteria to survive and grow in its environment. If a SNP is beneficial it can eventually be fixated in the population i.e. every individual in the population or subpopulation will have it. Also non-beneficial mutations can be fixated because of genetic drift. Anyhow the differences in accumulated SNPs are used to make phylogenetic inference on relatedness between individuals. The basic idea is that the more SNPs that have accumulated the longer time the organisms have been separated. In reality it is not that simple, mutations can reverse, occur at different rates and since there are only 4 bases, A, T, C and G, several mutations can sequentially happen and the final nucleotide could still be the same as the reference. Different mathematical substitution models have been developed for modelling purposes which assumes different patterns and rates, some examples are JC[122], HKY[123] and GTR[124] that are all commonly used in phylogenetic modelling[125].

For many years, phylogenetic inference was done on differences in single conserved genes in the organism/s that you wanted to examine. However, the introduction of WGS in surveillance now allows us to perform very fine-tuned phylogenetics on bacteria in outbreak situation utilising SNPs accumulated over the shared genomic parts between the isolates in an outbreak. It would not have been possible to analyse outbreaks with bacteria, with phylogenetics, using single conserved genes since not enough variation in form of SNPs exits to draw conclusion on relatedness close in time. In manuscript IV we performed a phylogenetic analysis of ESBL-producing *E. coli* ST131 using a SNP alignment from the shared genome of the dataset as input data.

Time-scale phylogeny, is a powerful tool in outbreak investigations since you can calculate the time point of the most recent common ancestor (MRCA). This means that you can determine approximately when an epidemic started and how it has spread from that time point. A story that demonstrated how powerful such an analysis can be is that of the "Bulgarian nurses affair" that took place in Libya from 1999 to 2007. Six foreign aid workers were imprisoned and sentenced to death after being accused of deliberately spreading HIV to more than 400 children in the hospital where they worked (http://news.bbc.co.uk/2/hi/europe/6192599.stm accessed on 2019-03-04). Suspicions were that this epidemic had already started before the aid works arrived in March 1998 and to corroborate this hypothesis, sequences from the hospital outbreak were compared to already published HIV sequences. The results of the time-scale phylogenetics showed that the strains from the children had a MRCA before 1998 and that the epidemic had already been spreading for quite a while before the foreign workers arrived to the hospital[126]. These scientific results together with other evidence made it possible for the European Union to feel secure when

negotiating for the release of the workers which eventually happened after almost 10 years of imprisonment.

Phylogenetics as a tool to investigate outbreaks is a relatively new field for public health microbiologists and still under development. Many questions remain to be answered, such as defining cut-offs on the number of SNPs that can be allowed to include isolates in an outbreak with a common source. Often straight and easy answers are demanded where there are none to be found. This is also the reason why I have chosen to make a more comprehensive description of this method.

1.8.1 Phylogenetic inference using Bayesian Evolutionary Analysis Sampling Trees (BEAST)

One way to perform time scale phylogeny is to use Bayesian inference. Bayesian statistics is separated from the classical frequentist statistics in several ways which is beyond the scope of this thesis. However, a fundamental difference relevant for the work within this thesis is that you use a prior probability distribution, referred to as just prior, that you draw samples from in order to make conclusions about the distribution in your data. Bayesian statistics was not popular during the 20th century but was revitalised toward the end of the 20th and beginning of the 21th century because of the introduction of the Markov Chain Monte Carlo (MCMC) algorithm. The MCMC allows sampling from the prior distribution, even if complex, in a computationally efficient way. The Monte Carlo refers to a stochastic method to generate numerical values from a probability distribution that is tested (e.g. an exponential population growth). The Markov Chain will link these stochastic draws so that the probability of a new event depends on the value of the previous event. The MCMC will generate a trace representing sampling events that is used to determine how well the data fits the tested prior.

BEAST is a software package that uses Bayesian inference and via MCMC infer time-scale phylogenetic trees, mutation rates and population sizes. To find the best fitting model for a dataset there are several different substitution models, molecular clocks and coalescent tree priors to select from[127,128].

Regarding molecular clocks, a strict clock is the most basic model, which assumes that the evolutionary rate is the same for all branches in the tree. Hence one clock parameter will be estimated[129]. A strict clock is always the basic parameter that is used to compare more complex models towards in order to estimate the best fit for the data. A more complex clock model is the uncorrelated relaxed clock, which allows the evolutionary rate to be different between the branches in the tree. This means that the evolutionary rate of one branch can be separated from the others. The relaxed clock therefore allows a more heterogeneous evolution where changes can happen fast in some lineages and slower in others[129].

The tree priors are used as the prior model that the MCMC samples from and they are all based on the theory of coalescence. The theory of coalescence refers to a model which traces

back alleles retrospectively to the last common ancestor, otherwise known as the most recent common ancestor (MRCA), for two individuals which have the allele under investigation. In the simplest model, the coalescent constant size prior assumes no population growth, no natural selection, no recombination and no gene flow which means that each allele is inherited to the next generation at a constant rate[127]. There is of course no natural population that behaves in this way but it is useful as a baseline for comparisons with other more complex tree priors. Other coalescent tree priors assume that some sort of population growth and/or decline has taken place, e.g. the exponential growth tree prior where we assume that natural selection has increased the frequency of the allele and/or the population. The different tree priors are used to see which of the models that fit the data best. For example, there are models referred to as non-parametric, e.g. Skyride and Skygrid, which use multiple time points to describe the dynamics of a population where several changes has occurred. These are helpful when modelling e.g. influenza epidemics or for epidemics where host immunity affects population size[130].

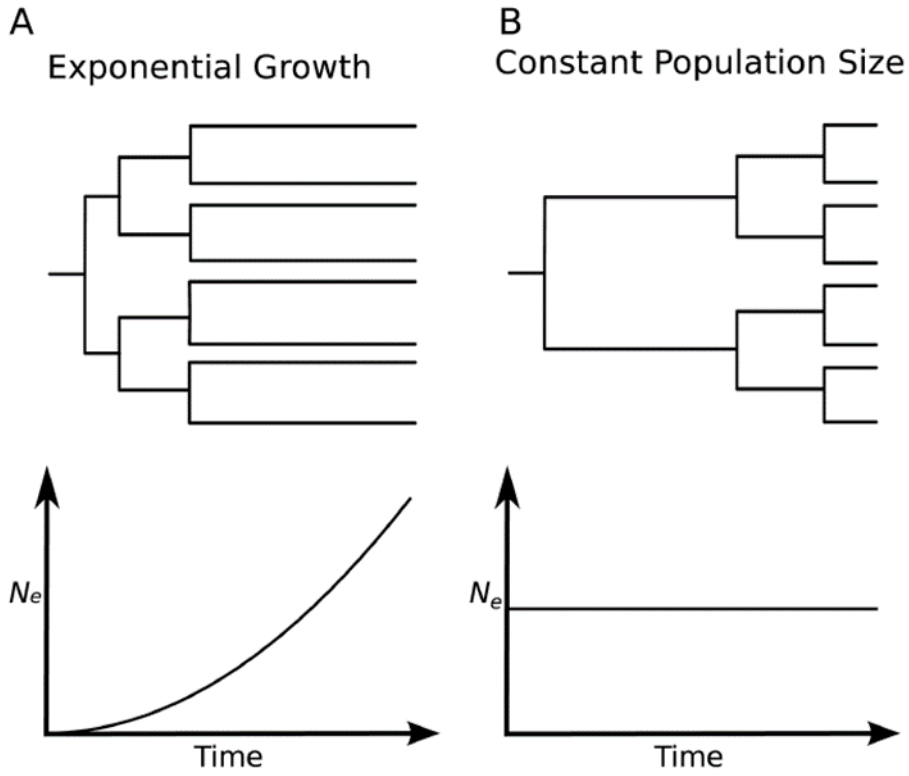

Figure 7. Schematic representation of population size over time in an exponential **(A)** and a constant **(B)** tree prior, copyright via CC BY 4.0[131]. The tree priors are used as models to test if the investigated population has spread in a similar manner.

When doing genealogies on bacteria that has recombinant regions, like *E. coli*, it is important to remove these regions prior to analysis. Failing to do so can disrupt the clock signal and lead to an overestimation of the evolutionary rate. Horizontally transferred genetic material could have evolved under a different selection pressure and therefore accumulated mutations.

When a recombinant region is introduced in a genome it can contain several SNPs, but it only represents one evolutionary event. This is also the basis of recognizing recombinant regions, you study parts of the shared genome that has a concentration of mutations compared to the reference genome or genomes[132].

2 AIMS

The general aim of the thesis was to investigate the epidemiology of ESBL/pAmpC-producing *E. coli* with a specific focus on community carriers and their role in the transmission chain. The prevalence of community carriers will affect the resistance burden on primary care and hospitals when an increasing share of patients that seek health care will be carrying ESBL/pAmpC-producing *E. coli*.

ESBL/pAmpC genes are widely disseminated globally in nature, among animals, in foods and in humans and we wanted to better understand what sectors and what type of ESBL/pAmpC-producing *E. coli* that contributes to sever clinical infections in humans. Our working hypothesis was that people that consume foods with high levels of ESBL/pAmpC-producing *E. coli* could become carriers of these bacteria in their intestine to a higher degree compared to people not consuming these foods to the same extent. Since intestinal carriage is a risk factor for clinical infection with ESBL/pAmpC-producing *E. coli* in humans[133,134], we further hypothesised that resistant bacteria from intestinal carriage also could contribute to severe infections. To answer these questions, we investigated the prevalence of ESBL/pAmpC-producing *E. coli* in different foods and compared the molecular characteristics of identified isolates to human isolates **(paper I).**

Since we wanted to study the epidemiology of ESBL/pAmpC-producing *E. coli* in community carriers we needed baseline information on how many in the community in general that carry these bacteria as well as information on important risk factors for being a community carrier. In **paper II** we aimed to investigate the general prevalence of ESBL/pAmpC-producing *E. coli* and *K. pneumoniae* in Sweden as well as risk factors associated with being a carrier. We hypothesized that consumption of chicken meat and foreign travel would be risk factors for being a community carrier.

In **paper III** we instead aimed to investigate the prevalence in specific study populations in Sweden and our neighbouring countries Finland, Latvia, Russia, Poland and Germany. We hypothesised that there would be differences in carrier prevalence between countries.

Many people are carriers without getting infections. Several patient risk factors, such as age and underlying diseases, make some patient more prone to get infections. However the pathogenicity of the *E. coli* bacteria might also play a role in why some get infections while others do not. We hypothesised that there are some types of ESBL/pAmpC-producing *E. coli* which more often cause clinical infections. We investigated this by comparing the molecular characteristics of ESBL/pAmpC-producing *E. coli* isolates from community carries and BSI **(paper II).**

In the comparison of molecular traits in **paper II** we identified several genetic characteristics that accumulated in BSI compared to carriers. Most of them were connected to the wide spread *E. coli* clone ST131. To understand the origin of the Swedish *E. coli* ST131 population we performed a comparative phylogenetic analysis with the international lineage in **manuscript IV**. Since the ST131 lineage is common in human infections knowing the

expected genetic variation is helpful when performing outbreak investigations. We hypothesised that several introductions from the international lineage would be present in Sweden. In addition, we expected that resistance genes would be located on both plasmids and chromosomes. We also wanted to investigate if one could detect similar plasmids in Swedish ST131 clusters, which would be helpful to guide future implementation of plasmid sequencing for transmission tracking.

3 METHODS

3.1 STUDY DESIGN AND SAMPLING METHODS

Several large national sample collections, from various sources, forms the basis of the data for the papers included in this thesis. The sample collections for paper I, II and IV were organised and carried out in a collaboration between the Public Health Agency of Sweden, the National Food Agency and the National Veterinary Institute. The collection of samples from foods (leafy greens and different kind of meats), farm animals and humans was coordinated both in time and geographically. Included non-human samples were leafy greens (n=522), meats (n=708) and farm animals (n=1082) (paper I).

In paper II we performed a population-based cross-sectional study to determine the prevalence of community carriers of ESBL/pAmpC-producing *E. coli* in Sweden in 2012-2013. A questionnaire based approach was used to collect information on possible risk behaviours associated with carriage of ESBL/pAmpC-producing *E. coli.* The study base was persons with a Swedish civil registration number between the age 18-72. In total 2134 individuals answered a questionnaire and provided faecal samples for screening. *E. coli*, with decreased susceptibility to cefotaxime and/or ceftazidime, from patients with BSI (n=418) were collected from Swedish clinical microbiological laboratories. Biases included response rate and recall bias.

In paper III we performed a cross-sectional study on the prevalence of ESBL-producing *E. coli* in defined cohorts (students, elective surgery, primary care visitors and web-based recruited volunteers) in Sweden, Finland, Latvia, Russia, Poland and Germany in 2015-2017. The cohorts were defined in order to collect comparable data between countries. A questionnaire was used to collect information on exposure to possible risk factors. The study base was people in the defined countries and cohorts between the age of 18-65 that had resided in the country for at least 1 year and not taken antibiotics during the last 3 months prior to sampling. For all participating countries 1211 faecal samples with accompanying questionnaire were collected in total.
Limitations included the use of different study populations in the participating countries, sample size, no information on response rates and recall bias.
The use of different sampling methodologies also makes it difficult to compare the Swedish prevalence results from paper II and III.

3.2 CLASSICAL TYPING METHODS

In our studies we have used both classical methods, such as phenotypic characterisation, molecular characterisation by PCR and Sanger sequencing of specific genes, as well as newer methods such as NGS of whole genomes. Phenotypic characterisation with disc diffusion and gradient tests were used on all isolates in our studies against extensive panels of antibiotics.

Furthermore, molecular characterisation was done with specific PCRs and Sanger sequencing to determine the MLST (Achtman) of isolates as well as the sequence of resistance genes.

3.3 NGS-METHODS

During the last five years molecular typing of antibiotic resistance has evolved from gene detection with PCR and Sanger sequencing to NGS based WGS, or amplicon sequencing, to detect regions coding for resistance, virulence factors or any other genetic element of interest as well as for MLST.

In this thesis we used long-read sequencing (PacBio) on a dataset of ESBL-producing *E. coli* ST131 (n=29). The PacBio methodology was developed by Pacific BioScience and is a single-molecule real-time (SMRT) sequencing method. The sequencing takes place in SMRT cells that contain wells called zero-mode waveguide (ZMW). In each well a DNA polymerase is fixated at the bottom which performs replication with fluorescent nucleotides. The DNA target is circularised by hairpin adaptors ligated to the ends, which allows multiple rounds of replication of a single target molecule. The long-reads allows for *de-novo* assemblies and can be used to map repetitive regions. It can also be used for transcript sequencing and provide information on base modulations such as methylation. The downsides of using PacBio is the high cost per base that is in part connected to the low throughput. It also has a higher error rate than short-read technologies[135]. We used PacBio in order to assemble plasmids and chromosomes fully (manuscript IV). The assembly allowed us to map the location of resistance genes within each genome which could be visualised together with the phylogenetic analysis of ESBL-producing *E. coli* ST131.

Some institutes, like the Public Health Agency of Sweden have created in-house pipelines to analyse gene content and the relatedness of isolates in a suspected outbreak. There are also several online tools that uses unprocessed short read data to analyse the presence of different genes as well as to perform phylogenies. One commonly used online tool to detect acquired antibiotic resistance genes, that we used, is ResFinder[136]. The tool allows you to upload raw reads from Illumina and Ion torrent or assembled genomes/contigs directly into a web application and then it identifies which resistance genes that are present in you data. The ResFinder database is based on resistance genes found in NCBI nucleotide database and is curated and updated regularly to include new resistance variants[136]. Historically ResFinder was focused on acquired resistance but since implementation of PointFinder it can also detect chromosomal point mutations known to be associated with antibiotic resistance[137]. In 2017 EFSA made a benchmarking study on different bioinformatics tools to detect AMR genes and concluded that several good tools exist. However none of the tools could detect chromosomal bla_{AmpC} mutations mediating β-lactam resistance[138].

The Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/ accessed on 2018-01-14) that harbours ResFinder also has additional detection tools and the repertoire is constantly expanding. At the time of writing (March 2019) they also supply analysis to detect plasmid

replicon type, perform MLST and plasmid MLST, find virulence or phage-associated genes, assemble genomes, conduct SNP analysis, and construct phylogenetics.

3.4 STATISTICS AND PHYLOGENETICS

We have used several statistical methods in our research but the two main methods were logistic regression, to analyse what risk factors that contributed to being a carrier of ESBL/pAmpC-producing *E. coli,* and Bayesian inference for the phylogenetic analysis. Logistic regression is used to model categorical dependent variables with e.g. a yes or no outcome, or in our case carrier or non-carrier of ESBL/pAmpC-producing *E. coli*. We use univariate and multivariate logistic regression to calculate odds ratios with confidence intervals for each of the risk factors we have asked our study subjects to report in questionnaires (paper II and III).

For the phylogenetic analysis we used the BEAST package to infer the *E. coli* ST131 genealogy (manuscript IV)[128,139]. In this analysis we used an international published *E. coli* ST131 dataset (Illumina) as comparison to our Swedish PacBio dataset[89]. An SNP alignment of the shared dynamic core genome between all isolates and the reference EC958[140] was used as input for the analysis. We used the shared genome to call SNPs from, instead of a cgMLST or wgMLST scheme, to achieve the highest possible resolution. Together the *E. coli* ST131 isolates shared 79% of their genomes, if we would have used a cgMLST scheme including 2000 genes as basis for SNP-call we only would have covered 30-40% of the genome. We also used SNP-call on plasmids in Swedish clusters to investigate the similarities in plasmid composition in clonal clusters. External reference plasmids were identified via BLAST at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 2019-03-26) using the assembled plasmids as input data. If no external plasmid was identified an internal was used as reference in the SNP-call.

3.5 ETHICAL CONSIDERATIONS

This thesis includes research with humans as study subjects. Ethical permission was sought and granted for each of the studies (registration numbers EPN 527/2012-4.1.2 and 2015/1893-31/1).

The most important ethical consideration when performing this type of research is the emotional impact on study participants when they find out that they are carriers of an antibiotic resistant bacterium. This can cause severe reactions and some individuals can take extreme measures to protect others from being exposed to their bacteria. Interview studies with carries show that some carriers alienate themselves and suffer severe anxiety[141]. Also, it could be difficult for carriers to come to terms with that nothing was done to eradicate the resistant bacteria and no follow-up screenings were offered.

To ensure that study participants could make an informed decision, information about the procedure and what it means to be a carrier were included in both the information before and after enrolment. The study participants that turned out to be carriers were given additional information and the opportunity to talk to one of our expert clinicians connected to the study either over phone or in person. We also adapted the information to make it easy to understand for the general public as well as objective and avoiding emotional statements that are often used in media to describe the problem with antibiotic resistance. Even though carriage of ESBL/pAmpC-producing *E. coli* is undesirable it is important to remember that it is common especially worldwide. We are, at least in some countries, in a situation where a large part of the population could be carries[59,117,142].

Recommendations for future studies is to include the possibility to get counselling since some participants were helped by this. It could also be an option to offer follow up screening for individuals who wish it, this is however complicated as we cannot say that you are not a carrier even if a sample is negative and that there are no guidelines regarding this in Sweden. Also, it is unclear how additional screenings would affect, or even help, the participant if they keep being positive carriers. Although most carriers lose their ESBL/pAmpC-producing *E. coli* after some time some individuals carry the bacteria for years, particularly those who have had a clinical infection[143].

Even though study participants can find it upsetting and challenging to receive the information that they are carriers of antibiotic resistant bacteria we still consider the studies justifiable. The studies contribute with information of public health importance regarding the epidemiology of ESBL/pAmpC-producing *E. coli* and *K. pneumoniae*. In addition, the majority of participants did not express any worries to us after receiving the message (although this does not mean that they did not worry). For the two Swedish studies around 2,400 individuals were enrolled resulting in around 120 carriers, of these two participants wished to get individual counselling with our expert clinician. Therefore, the extreme anxiety cases must be considered as quite rare events.

4 RESULT AND DISCUSSION

4.1 PAPER I

Limited Dissemination of Extended-Spectrum β-Lactamase- and Plasmid-Encoded AmpC-Producing Escherichia coli from Food and Farm Animals, Sweden.

Rationale

The motivation for performing this study was that more information was needed on how much of the ESBL/pAmpC-population that is shared between humans, foods and farm animals in Sweden. Without this information it is difficult to design effective prevention strategies to decrease the burden of ESBL/pAmpC-producing *E. coli* in human infections. If the transmission route of ESBL/pAmpC-producing *E. coli* to humans from foods like chicken meat is over- or underestimated this could lead to interventions without the desired effect of decreasing the burden of ESBL/pAmpC-producing *E. coli* in human infections.

This study was performed as a baseline for understanding how much resistance in farm animals and foods could contribute to humans becoming carriers and getting severe infections with ESBL/pAmpC-producing *E. coli*. We estimated the shared ESBL/pAmpC-producing *E. coli* population by studying basic molecular parameters such as type of ESBL genotype, plasmid replicon and plasmid MLST, and chromosomal MLST of the *E. coli* isolated from farm animals, meats, leafy greens, human community carriers and human BSI.

Main findings

Our main finding was that the population structure of ESBL/pAmpC-producing *E. coli* in foods and animals were mostly different from the population in community carriers and BSI. ESBL/pAmpC-producing *E. coli* isolated from mainly broilers, laying hens and chicken meat carried the ESBL genes $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ on different IncI1 and IncK plasmids. On the other hand isolates from humans mainly carried the ESBL genes $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ on different IncF plasmids. The *E. coli* MLST type in foods and animals was mainly ST10 while ST131 dominated among human isolates. *E. coli* ST10, IncI1plasmids and the genes $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-2}}$ were identified in both human, foods and farm animal isolates. Possible clonal overlap, i.e. the same *E. coli* ST, plasmid replicon and MLST and ESBL/pAmpC-gene between humans and animal/foods were found for one combination, ST155-IncI1 (ST3)- $bla_{\text{CTX-M-1}}$, in one community carrier and in one isolate from chicken meat.

Discussion

We conclude that the ESBL/pAmpC-producing *E. coli* found on farm animals, meats and leafy greens in Sweden have a limited effect on the prevalence of these bacteria in human community carriers and human BSI. The development seen within the human healthcare

sector, where the share of ESBL/pAmpC positive clinical *E. coli* infections have increased the last 10 years[68,70], is likely due to spread among humans. With that said we did identify some plasmids in isolates from both meats and humans that could potentially have disseminated from foods to humans but these plasmids were rarely identified in human BSI making the possible contribution from foods low. However, if plasmids and strains with a greater human pathogenicity becomes more prevalent in foods in the future they could serve as a potential reservoir for resistance plasmids and *E. coli* strains that cause clinical infections in humans.

Numerous studies on animals and foods as source for ESBL/pAmpC-producing *E. coli* have been performed, in different countries, with contradictory results and conclusions[113]. The reasons for this are multifactorial since different settings, animals, study designs and molecular methods were used. One example is studies in low- and middle-income countries where farming practices differs from the large-scale production we have in the western world[117,144]. There also seem to be a difference in how researchers interpret and angle their findings depending on if the scope of the study was to detect any transmission or to determine the overall level of contribution.

A recent joint source attribution study in the Netherlands analysing ESBL/pAmpC producing isolates from 22 different sources (e.g. humans, companion and farm animals, surface water, clinical infections, meats etc) isolated between 2013 and 2017, showed similar results as our study concluding that the contribution from the environment, animals and meats to humans were limited[145]. The exception were farmers that did have ESBL/pAmpC-producing *E. coli* with the same profiles as their livestock. In total, observations from 35 studies were included in this meta-analysis giving the result high external validity. Earlier studies, which suggested a large overlap of ESBL/pAmpC-producing *E. coli* between poultry and human clinical infections, likely overestimated the shared population. This because only basic genetics (gene, plasmid replicon and *E. coli* MLST) were used to draw the conclusions[96,111].

One can only speculate in why we do not become carriers or get infections from poultry associated ESBL/pAmpC-producing *E. coli* to a higher extent. One reason could be that the animal-associated *E. coli* types that carry the ESBL/pAmpC-genes are not adapted to colonise the human intestine and lack the necessary virulence factors to cause human infections. If the *E. coli* cannot colonise our intestine the opportunity for plasmid spread would also be limited. However, more research is needed to establish these connections.

The high prevalence of ESBL/pAmpC-producing *E. coli* in poultry and chicken meat on the Swedish and international market is still highly problematic since it forms a reservoir of resistance that is difficult to get rid of. It is possible that if the molecular epidemiology of *E. coli* in poultry changes to more human adapted *E. coli* the contribution could be greater in the future. One example is the emergence of ST131 reported in poultry[118].

4.2 PAPER II

Community carriage of ESBL-producing Escherichia coli is associated with strains of low pathogenicity: a Swedish nationwide study.

Rationale

The motivation to perform this study was to investigate the nationwide prevalence of ESBL/pAmpC -producing *E. coli* and *K. pneumoniae* carriers in the general community and risk factors associated with becoming a carrier. Knowing the community prevalence gives us better understanding of how widely disseminated ESBL/pAmpC are in the Swedish community and forms baseline data to compare to in the future. Risk factors and behaviours associated with carriage on a community level is valuable to know in order to adjust recommendations and interventions for different purposes like indications for taking screening samples before hospitalization.

Clinical infections like UTI with ESBL/pAmpC-producing *E. coli* will usually be preceded by intestinal carriage[133,134,146]. However, depending on the pathogenicity of the *E. coli* that harbour the ESBL gene some patients might be at higher risk for acquiring an UTI which in turn could lead to more severe infections such as BSI. We wanted to identify bacterial characteristics that were associated with BSI in humans by comparing molecular characteristics of ESBL/pAmpC-producing *E. coli* isolated from carriers and BSI.

Main findings

We found that the prevalence of ESBL/pAmpC-producing *E. coli* carriers in the Swedish community population was 4.7%. No carriers of ESBL/pAmpC-producing *K. pneumoniae* were identified. Independent risk factors, from the multiple regression analysis, for being a carrier were travel to Asian and African countries the last 6 months and a diet that excluded pork meat.

Characteristics connected to pathogenicity of the *E. coli* bacteria, phylogenetic group B2, ST131 and its subclone C2 (called H30-Rx in paper II), were overrepresented in BSI compared to community carriers where the share of these traits were lower. The ESBL genotype $bla_{\text{CTX-M-15}}$ was associated with BSI while the genotypes $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-1}}$ were associated with community carriers. Also, higher levels of clinically relevant phenotypic resistance were associated with BSI.

Discussion

The prevalence of Swedish ESBL/pAmpC-producing *E. coli* at 4.7% will be a useful baseline number for future prevalence studies. Travelling to high prevalence regions was identified as a risk factor also on a community level, which was expected since this has been seen in other studies on travellers before and after travel[54,55,61]. The result that having a diet that included pork were protective against being an ESBL carrier was a surprising since there is no obvious

biological connection. Having a diet that included chicken was not a significant risk factor which was also surprising since we know that chicken meat on the Swedish market often contain ESBL/pAmpC-producing *E. coli*[114]. We therefore conclude that the non-pork eaters, which had a higher risk, have something else in common that we did not measure e.g. travel to high prevalence regions further back in time than 6 months.
Our results clearly showed that some molecular characteristic connected to the pathogenicity of the *E. coli* were overrepresented in isolates from BSI compared to carriers. This suggests that it might be clinically relevant to categorize carriers as high- or low-risk depending on what type of *E. coli* strain that they carry. This might be useful in future scenarios if the community prevalence increases, or in places where the community prevalence is already high, and not all positive ESBL/pAmpC carrying patients can be isolated. We also saw that the globally spread *E. coli* ST131 subclone C2 was present in both carriers and BSI. This subclone alone cause an estimated 26% of all BSI infections with ESBL/pAmpC-producing *E. coli* in Sweden. This is a very large burden caused by one single clone which prompts further investigation into the connection between the Swedish and global epidemiology of the C2 lineage.
The patient group that get BSI is mainly elderly patients often with several comorbidities. It is therefore possible that the accumulation effect was partially due to a circulation of more pathogenic strains among vulnerable patients in e.g. hospitals and long-term care facilities. A multi-resistant strain like ST131 would certainly have an advantage in these types of environments where the selection pressure from antibiotics would be high. A screening study aimed at this patient group would therefore be an interesting addition and add information regarding the population structure of ESBL/pAmpC producing *E. coli* in high-risk groups.

It is difficult to find studies to compare our results to because i) few studies on community carriers have been performed and ii) most studies on risk factors for BSI focuses on patient factors such as different comorbidities, antibiotic consumption and presence of devices like urine catheters[134,146,147]. Studies performed on cohorts representing the community from other European countries reported prevalences close to 6% which is in a similar range as the 4.7% identified in our study[56,148-150].
A large screening study performed on persons with close contact to gastroenteritis patients in Germany from 2009 to 2012 showed similar distribution of ESBL genes with $bla_{\text{CTX-M-15}}$ being the most common[148]. In addition meta-analysis clearly showed that the $bla_{\text{CTX-M}}$ genes are the dominating ESBL genes in carriers globally which further supports our findings[59].

To conclude both collections (carriers and BSI) used to compare molecular characteristics are large and representative for the entire population in Sweden which is why we consider the results to have high external validity.

4.3 PAPER III

Large variation in ESBL-producing Escherichia coli carriers in six European countries including Russia.

Rationale

The motivation to perform this study was to collect baseline data on the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* carriers in Northern Europe represented by Finland, Latvia, Russia, Germany, Poland and Sweden. Carriers have a central role in the epidemiology of ESBLs and previous studies show that the prevalence can vary a lot depending on which population and which country you study. Travel to high prevalence countries is a known risk factor for becoming a carrier and much of the travels we make are to our neighbouring countries[54,55,61]. Even though carriers have an important role in the transmission of ESBLs as a link between environment, community and clinical infections structured surveillance are missing in both national and international initiatives. We performed a prevalence study on specific study populations, screened for ESBL-producing *E. coli* and *K. pneumoniae* using the same methods, in Finland, Latvia, Germany, Poland, Russia and Sweden. Capacity building to improve the ability to perform studies on carriers was a secondary aim of the study.

Main findings

Our main finding was that the prevalence varied between the investigated countries. It was highest in Russia (23.5 %) and lowest in Latvia (1.6%). The other countries in the study had prevalences of 2.3% for Germany, 4.7% for Finland, 6.6% for Sweden, 8.0% for Poland summarizing to 8.1% for all countries in total. Carriers of ESBL-producing *K. pneumoniae* were identified in three of the six countries, Finland, Russia and Sweden, but in low numbers (<2%). None of the countries identified carriers of carbapenemase-producing *E. coli* or *K. pneumoniae*. The study populations varied somewhat between countries and included students, primary care visitors, elective surgery patients, and volunteers recruited via a website. A risk analysis was performed from the questionnaires that each study participant answered. Positive risk factors were only seen for Russian study subjects where the age groups 31-50 and 51-65 were at higher risk of being carriers compared to 18-30 years. In addition, hospitalization the last 6 months was also a risk factor for being a carrier of ESBL-producing *E. coli* in Russia.

Discussion

Our results suggest that there could be a large difference in carrier prevalence of ESBL-producing *E. coli* in neighbouring countries in the Northern Europe. The most notable difference was that between the prevalence in Russia and Latvia at 23.5% vs 1.6% respectively. We cannot exclude that the difference was due to study population differences with variations in underlying risk factors. However, nothing in the baseline characteristics

suggested that this was the case since the study populations in Russia and Latvia had similar mean age, antibiotic consumption history and hospitalization proportions. It could be that our results reflected two different country situations where Latvia has not had a wider introduction of ESBL-producing *E. coli* in their community. This could be due to differences in historic antibiotic use and what type of antibiotics that were commonly used.
The Swedish result with a prevalence of 6.6% was slightly higher but in a similar range compared to our earlier study that gave a prevalence of 4.7%. Since different collection strategies and study populations were used the numbers are not directly comparable.
Very few carriers of ESBL-producing *K. pneumoniae* were identified even in population with high prevalence of ESBL-producing *E. coli* which indicates that these are not common in the community. In addition, we did not identify a single carbapenemase-producing isolate which also indicates that these are not widely disseminated in carriers in our study populations yet.
A recent large study on asylum seekers, from countries suspected to have a high prevalence of both ESBL/pAmpC and carbapenemase-producing *E. coli*, did not identify any carbapenemase-producing isolates[65]. This strengthens our conclusion that these bacteria are still not widely disseminated in the community.
In conclusion, more studies are needed on study populations representing the community in order to establish that the differences seen in our study are valid.

4.4 PAPER IV

Genome and plasmid diversity of ESBL-producing Escherichia coli ST131 – tracking phylogenetic trajectories with Bayesian inference.

Rationale

We performed this study to investigate the ESBL-producing *E. coli* ST131 population and its subclones in Sweden more closely. As concluded in paper II, ST131 and its subclone C2 are responsible for a large proportion of Swedish BSI with ESBL-producing *E. coli*. To better understand their emergence, epidemiology and resistance gene structure could be important to detect and design interventions towards this problematic lineage. The aim of the study was to determine if the Swedish ESBL-producing *E. coli* ST131 population originates from single or multiple introductions from the globally disseminated clone. This would help in e.g outbreak investigations since it is at the moment hard to know what amount of variation that can be expected within the different ST131 clonal groups. We also wanted to investigate the relatedness of plasmids in identified clusters to see if single or multiple plasmids co-evolve with some of the clones.

In order to get a better view of the location of the resistance genes within the genome and to obtain fully assembled plasmids, we used PacBio long-read sequencing on a subset of isolates from the collections of BSI and community carriers collected in paper II. We also used an

external published dataset of international *E. coli* ST131 strains as comparison for the phylogenetic analysis[89].

Main findings

We found that the Swedish ESBL-producing *E. coli* ST131 were distributed in the three previously identified clades A, C1 and C2 in the international ST131 phylogeny. The majority of our isolates belonged, as expected, to the C2 lineage. We identified three Swedish clusters, with a MRCA, in the A, C1 and C2 clades. Plasmid SNP calling amongst plasmids in Swedish transmission clusters displayed conserved plasmid lineages in all clades as well as some completely unrelated plasmids in clade A. The full assembly and mapping of resistance genes, to plasmids and/or chromosomal regions, for each strain revealed a heterogeneous distribution of the resistance genes that were often found in two locations in the genome either in two different plasmids or in one plasmid and in a chromosomal region.

Discussion

Our main finding was that multiple introductions have shaped, and continue to shape, the Swedish population of ESBL-producing *E. coli*. With that said, we did not quantify the national versus international dissemination and their respective contribution to the burden of infections caused by ESBL-producing *E. coli* ST131 in Sweden. Even though continuous introductions contributed to infections it is very possible that the greatest burden is caused by local dissemination, especially among vulnerable patient groups.

The BEAST analysis supports the previous conclusion that the ST131 clade C2 increased exponentially during the 1990s and after that reached a plateau[86]. This emergence pattern coincided with the introduction of the fluoroquinolone ciprofloxacin in the late 1980s which supports the conclusion of several experts and studies that fluoroquinolone resistance has been a major reason for the successfulness of ST131[81,82,86,88].

Isolates from especially two ST131 lineages, clade C2 with $bla_{\text{CTX-M-15}}$ and clade C1 with $bla_{\text{CTX-M-27}}$, circulating in Sweden are genetically very similar. This was not a surprising result since we know that they originate from single clones which emerged not long ago[86,88,89]. However, it complicates outbreak investigations with these lineages since it makes it hard to separate direct transmission from isolates that are just part of the clonal lineage. In for example the C1 transmission cluster the Swedish isolates were likely separated from a MRCA 10 years ago and differ from each other with around 30 SNPs.
In the case of the C1 clade with $bla_{\text{CTX-M-27}}$ we also identified a highly conserved plasmid in all isolates, which very likely has co-evolved with the lineage for at least 10 years. This was a major difference to the C2 lineage where several different IncF plasmids were identified in our data. This could be due to that we have a limited number of isolates in total and more isolates from the C2 clade compared to the C1 clade. However, previous studies on ST131 and the C2 clade have also concluded that several plasmids are associated with this clade[76,151]. Further investigation is needed to determine reasons for the strong co-evolution of

the IncF plasmid in the C1 *bla*$_{\text{CTX-M-27}}$ clade. Possible explanations could be a potent toxin-antitoxin system or an early loss of mobility so that the plasmid cannot move horizontally. One reason could also be that the selective pressure from antibiotics acts only on that particular plasmid if the *bla*$_{\text{CTX-M-27}}$ is not mobile by connection to an active transposon, like *bla*$_{\text{CTX-M-15.}}$ This could also explain the theoretical lack of co-evolving plasmids in the C2 clade since *bla*$_{\text{CTX-M-15}}$ is connected to an active *ISEcp1* transposon and therefore move to both chromosomal regions and other plasmids[76,77]. It would be interesting to further investigate these theories, as well as a larger dataset of the C1 *bla*$_{\text{CTX-M-27}}$ clade to confirm if the co-evolving plasmid is present in the whole or just a sub-set of the clade.

As hypothesised we found resistance genes in both plasmids and chromosomal regions. All chromosomal integrations were connected to the *bla*$_{\text{CTX-M-15}}$ gene. Previous theoretical modelling suggested that chromosomal integration, of beneficial plasmid genes, should be evolutionary favoured under high selection pressures since the cost for carrying the plasmid would be evaded[152,153]. However most of our isolate with chromosomal integrations also carried resistance plasmids with the same or additional resistance genes.

These highly pathogenic and common clonal lineages are important to monitor by national surveillance since they have a very high propensity to accumulate resistance genes and are under constant selection pressure from the antibiotics we humans consume. In a not too unlikely scenario these clones will acquire resistance to last resort drugs such as carbapenems and colistin, which will increase the burden on vulnerable patient groups and healthcare even more.

5 CONCLUSION

This thesis investigated the epidemiology of ESBL/pAmpC-producing *E. coli* with a focus on describing possible sources and risk factors for human community carriage. Furthermore we investigated which traits that were overrepresented in isolates from BSI compared to carriers to identify what defines high-risk pathogenic isolates. In addition we performed a study of the emergence of the particularly problematic ESBL-producing *E. coli* clone ST131, in Sweden.

The overall conclusion from this work is that human to human transmission of ESBL/pAmpC-producing *E. coli* is the main contributor to the burden of infections in humans with these bacteria. The results from our study suggests that the contribution to severe infections in humans from especially the poultry sector, where high prevalence of ESBL/pAmpC is detected, is limited.

Community carriage of ESBL/pAmpC producing *E. coli* was around 5% in Sweden and we identified large variation in carrier prevalence in our neighbouring countries around the Baltic Sea. For Swedish carriers we saw that travel to high prevalence regions was a major risk factor for being a carrier of ESBL/pAmpC producing *E. coli* on community level. We did not identify carriers of carbapenemase-producing *E. coli*, in any of our studies.

We concluded that there was an overrepresentation of the most pathogenic *E. coli* isolates in BSI compared to carriers, where the most dominant type was ST131. Phylogenetic modelling on Swedish ESBL-producing *E. coli* ST131 showed that several introductions from the international epidemic have shaped, and continuously shape, the Swedish population. However, the main contributing factor to infections with ESBL/pAmpC producing *E. coli* in Sweden could still be, and likely is, national dissemination.

Overall, our studies contribute to a deeper understanding of how ESBL/pAmpC-producing *E. coli* circulate in Sweden and what factors that contribute to increased carrier prevalence and severe infections with these bacteria. This type of epidemiological surveillance is necessary in order to design efficient intervention strategies with the aim to reduce clinical infections with ESBL/pAmpC in humans.

6 FUTURE STUDIES ON CARRIERS

It is challenging to study community carriage of ESBL/pAmpC-producing *E. coli* and other species for several reasons. First, it is difficult to reach a study population that is representative for the general community. Second, response rates are usually low which might give a skewed representation since we do not know why some participants decline to take part in the study. These factors affect both the internal and external validity of the results. Often the focus is on having a high number of participants in order to achieve enough power to make the results valid. Although power is an important factor it is often the poor representativeness, of the population you want to draw conclusions about, that is the main issue.

One way to get around this could be to stop focusing on generalizing findings to the community and put more effort towards investigating specific vulnerable groups such as long-term care facility residents. A key factor to increase the response rate is that you cannot rely on the participants goodwill, but have to offer something in return for their participation. Another interesting study would be to perform prospective cohort studies to determine the incidence in specific populations. In the case of carriers there would be two rates that are interesting to study, 1) the rate of new cases in a population and 2) the rate of loss of cases in the same population. However, it is important to consider the benefits and what is gained by investigations such as these since they are often demands a lot of resources and could be ethically challenging.

7 POPULAR SCIENCE DESCRIPTION

Djuren, människan och antibiotikan

De senaste åren har antibiotikaresistens seglat upp på dagordningen hos politiker och beslutsfattare världen över. Anledningen; antibiotikaresistensen ökar snabbt och något måste göras för att hindra dess framfart. Men hur sprids antibiotikaresistens och vilka faktorer är det som ligger bakom ökningen av resistenta infektioner?

Antibiotikaresistens är ett komplext problem som involverar många olika aktörer såsom sjukvården, jordbruket, reningsverken, läkemedelsbolagen, läkemedelstillverkarna samt dig och mig. Det finns idag ett flertal sjukdomsframkallande bakterier som har utvecklat alvarliga former av resistens såsom *Mycobacterium tuberculosis* och *Neisseria gonorrhoeae* som orsakar tuberkulos respektive gonorré.

Den här avhandlingen handlar dock om en annan typ av antibiotikaresistens som kallas ESBL (Extended-Spectrum β-Lactamase). ESBL syftar på en grupp med gener som kodar för resistens mot β-lactam antibiotika med utvidgat spektrum. Bakterier som har en ESBL gen blir därför resistenta mot viktiga antibiotika som penicilliner och cefalosporiner. Eftersom ESBL gener kan spridas mellan bakterier kan en uppsjö av olika bakteriearter bära på ESBL. En av de mest problematiska ESBL-bildande bakterierna är *Escherichia coli* som kan orsaka urinvägsinfektioner och blodinfektioner. År 2017 orsakade ESBL-bildande *E. coli* ca 100 infektioner per 100 000 invånare i Sverige vilket är en kraftig ökning från 40 infektioner per 100 000 invånare år 2009[154].

ESBL-bildande *E. coli* har en fekal-oral spridningsväg och när vi får i oss bakterierna blir vissa av oss bärare, dvs de stannar kvar ett tag i vår tarmflora. Därifrån kan bakterierna orsaka infektioner som människor är olika mottagliga för beroende på vår allmänna hälsostatus och vårt immunförsvar samt hur benägen *E. coli*-bakterien är att orsaka infektioner. De allra flesta som är bärare vet inte om det och påverkas inte heller av sitt bärarskap. Dock bidrar bärarna med att sprida bakterier vidare till andra människor och miljöer vilket ökar utbredningen av resistenta bakterier i samhället. Man kan därför se bärarna som en form av indikator för hur spridda ESBL-bildande *E. coli* är i samhället.

I två av studierna i denna avhandling undersökte vi andelen bärare i Sverige samt i våra grannländer runt Östersjön; Finland, Lettland, Ryssland, Tyskland och Polen (**artikel II** och **III**). I Sverige bär ca 5% av befolkningen på ESBL-bildande *E. coli* och en riskfaktor för att bli bärare är att resa till länder i Asien eller Afrika.

Årligen gör svenskarna ca 200,000 resor till Thailand[66]. Enligt tidigare studier är ungefär 20-30% av resenärer till sydöstra Asien bärare av ESBL-bildande bakterier när de kommer hem[54,55,61]. Detta beror på att länder som Thailand, Indien och Kina har en högre förekomst

av ESBL-bildande *E. coli* i samhället som vi sannolikt får i oss via kontakt med människor, föremål och livsmedel.

Tidigare studier har visat att ESBL-bildande *E. coli* är vanliga inom kycklingproduktionen såväl i Sverige som i resten av världen. Kycklingarna bär bakterien i sina tarmar och när de slaktas kontamineras köttet som vi sedan äter. Ungefär 40% av all kyckling i svenska matbutiker är kontaminerad med ESBL-bildande *E. coli*. Enligt Jordbruksverket konsumerade Svensken år 2017 23,2 kg kyckling per person[155]. Alltså konsumerar vi regelbundet en hel del ESBL-bildande *E. coli* från djur.

Vi vet alltså från våra och andras studier att vi får i oss och bär på ESBL-bildande *E. coli* från olika källor. Men är det samma bakterier på kycklingköttet som vi får alvarliga infektioner av? För att ta reda på detta gjorde vi en genetisk jämförelse mellan isolat av ESBL-bildande *E. coli* från djur och livsmedel med de från bärare och bakterier isolerade från blodinfektioner (**artikel I**). Resultaten visade tydligt att olika typer av genetiska profiler dominerar hos livsmedel (främst kyckling) jämfört med människor (bärare och blodinfektioner). Vi kan alltså inte skylla den ökande andelen ESBL i infektioner hos människor på att det finns en hög andel ESBL-bildande *E. coli* på kyckling.

När vi genetiskt jämför ESBL-bildande *E. coli* isolat från bärare med blodinfektioner ser vi att en viss typ av speciellt sjukdomsframkallande *E. coli*, kallade ST131, är vanligare i blodinfektioner (artikel II). Denna typ av *E. coli* bakterie är spridd globalt och vanlig i infektioner världen över. När vi undersökte den genetiska variationen hos den ESBL-bildande *E. coli* ST131 populationen i Sverige (**manus IV**) såg vi att den introducerats flera gånger i Sverige från den globalt spridda ST131 populationen. Troligen sker introduktionen via resenärer som är bärare och sedan sprider bakterien vidare till sjukvården och individer som är mottagliga för blodinfektion.

Sammantaget visar forskningen i denna avhandling att riktade insatser för att minska spridning mellan människor är den viktigaste åtgärden för att minska andelen ESBL-bildande *E. coli* i infektioner. Särskilt sjukdomsframkallande typer av ESBL-bildande *E. coli* som orsakar en stor andel infektioner behöver övervakas specifikt. I dagsläget är det inte troligt att vi kommer minska andelen ESBL-bildande *E. coli* i alvarliga infektioner hos människa genom att minska andelen av liknande bakterier hos kyckling. Det är dock fortfarande problematiskt och icke önskvärt att vi har en hög andel ESBL-bildande *E. coli* på framförallt kycklingkött bland annat eftersom det skulle kunna ske skiften till mer sjukdomsframkallande bakterier. I så fall kan denna spridningsväg få en mycket större klinisk betydelse.

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